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Signal Transduction Mechanisms Involved in Hormonal Ca^{2+} Fluxes

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This article reviews literature up to mid-1988 covering recent developments pertaining to agonist-induced Ca^{2+} signaling in various cell types. A large amount of experimental evidence supports a mechanism involving specific guanine nucleotide-binding proteins (G-proteins) as transducing factors between occupancy of a wide variety of receptors by many different agonists and activation of polyphosphoinositide specific phospholipase C enzymes. Although many different G-proteins and phospholipase C enzymes have been purified and cloned, successful reconstitution of the components has not been achieved. Hence, many questions concerning the specificity of coupling between particular receptors to a particular G-protein and phospholipase C subtype remain unresolved. Phospholipase C subtypes isolated from the membrane and soluble fractions of the cell are directly activated by Ca^{2+} and, preferentially, hydrolyse phosphatidylinositol 4,5-*bis*phosphate (PIP_2) and phosphatidylinositol 4-phosphate (PIP). The role of the G-protein is to stimulate inositol lipid breakdown at free Ca^{2+} concentrations (0.1–0.2 μM) typical of unstimulated cells. Overwhelming evidence supports the concept that Ins 1,4,5-P_3, the product of PIP_2 hydrolysis, is responsible for the initial agonist-induced Ca^{2+} transient by mobilization of Ca^{2+} from a specialized intracellular store. An Ins 1,4,5-P_3 receptor has been purified that may correspond to the postulated Ins 1,4,5-P_3 gated Ca^{2+} channel. Despite a growing understanding of the complexities of the metabolism of Ins 1,4,5-P_3 and a successful purification of many enzymes involved, including the ATP-dependent 3-kinase that converts Ins 1,4,5-P_3 to Ins 1,3,4,5-P_4, the role of Ins 1,3,4,5-P_4 as a putative second messenger remains enigmatic. Multiple forms of protein kinase C have been described and the role is well established for a 1,2-diacylglycerol, the second product of PIP_2 hydrolysis, as its physiological activator. Although protein kinase C has been shown to phosphorylate and modulate the activity of several proteins involved in the Ca^{2+} signaling pathway and Ca^{2+} transport, the physiological significance of the protein kinase C in agonist-stimulated cell function requires further elucidation. The extension of measurements of hormone-induced Ca^{2+} changes to single cells has shown that the occurrence of Ca^{2+} oscillations is a common phenomena. Elucidation of the biochemical mechanisms causing this oscillatory response and its physiological significance represents an important challenge for future studies.

Introduction

The cytosolic free Ca^{2+} concentration functions as an important intracellular signaling mechanism whereby hormones and growth factors regulate many different cellular processes such as secretion, metabolism, neurotransmitter release, cell growth, and differentiation. Signal transduction by the ligand-activated receptor is mediated by specific guanine nucleotide binding proteins (G-proteins), which activate phospholipase C-mediated hydrolysis of phosphatidylinositol 4,5-*bis*phosphate (PIP_2) in the plasma membrane. The products of this reaction are 1,2-diacylglycerol, which is retained in the plasma membrane, and D-*myo*-inositol-1,4,5-trisphosphate (Ins 1,4,5-P_3). Both compounds act as intracellular messengers with different functions.

Ins 1,4,5-P_3 is responsible for mobilizing intracellular Ca^{2+}, thereby causing a rapid increase of cytosolic free Ca^{2+}. This is transient because of an activation of Ca^{2+} efflux mechanisms in the plasma membrane—notably the Ca^{2+}-ATPase. In addition, receptor occupancy is associated with an enhanced influx of Ca^{2+}, which maintains the cytosolic free Ca^{2+} above resting levels for the duration of the agonist response (*1,2*).

Diacylglycerol activates a Ca^{2+} and phospholipid-dependent kinase, termed protein kinase C, and promotes its translocation to the plasma membrane (*3,4*). Protein kinase C has short-term modulatory effects mediated by protein phosphorylation at the level of the receptor-G-protein complex and at post receptor sites involving Ca^{2+} transport and Ins 1,4,5-P_3 metabolism. It also has long-term effects on secretion and the regulation of the expression of a number of

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gene products involved in cell growth (*5,6*). These latter effects may be mediated by protein kinase C isozymes that are different from those responsible for the short-term effects (*7–9*).

Despite a general acceptance of the overall sequence of events responsible for linking receptor activation to increases of cytosolic free Ca^{2+} (*10–13*), many details of the mechanisms underlying each step remain unresolved. Recent advances suggest a high level of cellular diversification and the presence of an intricate network of positive and negative controls providing continuous regulation of the response elicited by receptor activation.

This review will describe recent findings concerning various types of novel G-proteins and phospholipases found in different cells, and the mechanisms involved in regulation of Ca^{2+} flux across the plasma and intracellular membranes. These mechanisms, together with a variety of feedback controls, could account for diverse cellular Ca^{2+} responses seen in different tissues, as well as the marked heterogeneity of response seen in single cells within a population, including an oscillatory behavior of the Ca^{2+} signal.

Generation of Intracellular Second Messengers

The feature that appears to be common to different cell types for agonist-induced Ca^{2+} mobilization involves an interaction in the plasma membrane between three different types of proteins, namely, receptors, G-proteins, and phospholipase C. Most cells contain many different types of receptors in the plasma membrane which, upon stimulation by suitable agonists, cause an increase of cytosolic free Ca^{2+} (*10,14–16*). The number of receptors and their affinity determines the relative sensitivity of the cell to each agonist and the extent of phospholipase C activation. Since a particular agonist is often capable of interacting with several different subtypes of receptors, the specificity of signal transduction is likely to be determined by the nature and properties of the G-protein that interacts with a particular receptor subtype and effector system (*17*).

The Role of G-Proteins in Activating Phospholipases

The structure and molecular properties of G-proteins and their role in signal transduction have been reviewed recently (*18,19*). Those involved in receptor coupling in the plasma membrane act as both on/off switches and a mechanism for signal channelling and amplification. Generally they are present in greater abundance than the receptor proteins. G-proteins typically have a heterotrimeric αβγ structure. The α-subunit is the site for GTP-binding, and differences in their primary structure (*20–23*) provides the basis for their specific roles as transducing agents. A general model for G-protein action has been proposed, which is based on numerous studies concerned with the mechanism of activation of adenylate cyclase and photoreceptor function (*18,24*). Binding of agonist to the receptor causes the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) on the G-protein α-subunit. This is accompanied by a dissociation of the α-subunit from the heterotrimer and a decreased binding affinity of the agonist to the receptor. The activated (GTP-bound) α-subunit then interacts with and modifies the activity of the effector protein. The G-protein α-subunits have an intrinsic GTPase activity, yielding inactive α-GDP complexes, which reassociate with the available pool of βγ-dimers to reform the heterotrimeric G-protein, which can then undergo further cycles of activation-deactivation with signal amplification. Different forms of β- and γ-subunits also exist, but the primary role of the more lipophilic βγ complexes, or myristoylated α-subunits, (*25*) might be to anchor the G-protein in the membrane, possibly adjacent to a particular receptor subtype.

Several properties of the G-proteins provide useful tools for investigating their functional role in cellular processes. Nonhydrolyzable GTP analogues such as GTPγS cause a persistent activation of G-proteins, but GDPβS, which mimics GDP, is inhibitory. Another property of the G-protein α-subunits is their susceptibility to ADP-ribosylation by bacterial toxins. Cholera toxin catalyses ADP-ribosylation at an arginine site of several G-proteins, and pertussis toxin causes ADP-ribosylation at a cysteine site, located near the carboxyl terminus, and uncouples the G-protein from the receptor. The α-subunits of different G-proteins may be ADP-ribosylated by cholera or pertussis toxins (*18*). Notably, the G-protein responsible for stimulating adenylate cyclase (Gs) is a substrate for cholera toxin, while the G-protein associated with inhibition of adenylate cyclase (Gi), and a G-protein prevalent in brain (Go) are substrates for pertussis toxin.

The original evidence leading to the conclusion that G-proteins are involved in receptor coupling of Ca^{2+}-mobilizing agonists to PIP_2 breakdown was reviewed recently (*12,19,26,27*). The basic observations are that G-protein activating agents such as GTP, GTPγS or AlF_3 (which mimics GTP binding) when added to permeabilized cells or plasma membrane preparations cause an activation of phospholipase C and a decrease of agonist binding affinity. Further evidence is provided by *a*) the enhancement of phospholipase C activation by suboptimal concentrations of GTPγS in the presence of agonist, *b*) an agonist-induced stimulation of GTPase activity, *c*) the inhibition of phospholipase C activation by GDPβS, *d*) an inhibition of agonist-induced activation of phospholipase C by toxins (some cell types only), and *e*) the retention of receptor-G-protein complexes (*28–30*) or G-protein-phospholipase C complexes (*31*) during purification.

Current evidence suggests that a variety of different types of G-proteins are responsible for regulating receptor coupling to phospholipase C in different cell types. This was first suggested by the fact that agonist-mediated stimulation of inositol phosphate formation and Ca^{2+} mobilization could be inhibited by pertussis toxin pretreatment in some cell types, but not others [for references and details of cell types see (*26*)]. Ca^{2+} mobilization induced by activation of different receptors can also be selectively inhibited by pertussis toxin pretreatment in the same cell type. In hepatocytes, epidermal growth factor (EGF) responses are pertussis-toxin sensitive, whereas those to vasopressin or angiotensin II are not (*32*). In platelets, pertussis toxin inhibits thrombin but not U46619 (a thromboxane A_2 analogue) responses (*33*). Further, heterogeneity in the type of G-protein able to couple to phospholipase C is suggested by the ability of cholera toxin to inhibit the effects of Ca^{2+}-mobilizing agonists in a variety of cell types (*27,34-37*). This effect of cholera toxin was independent of ADP-ribosylation of G_s and changes of cyclic AMP.

A number of novel G-proteins that may be involved in coupling to phospholipase C have been identified in various cells. In neutrophils and human leukemia HL-60 cells (*30, 38-40*), a pertussis toxin-sensitive 40 kDa α-subunit has been purified and shown to be immunologically distinct from the α-subunits of Go and Gi. The copurification of this 40 kDa polypeptide with the chemoattractant receptor suggests that it is responsible for activation of phospholipase C (*30*). This same 40 kDa α-subunit from HL60 cells is ADP-ribosylated by cholera toxin in the presence of the Ca^{2+}-mobilizing chemotatic peptide fMet-Leu-Phe, as well as by pertussis toxin (*34,35*).

A pertussis toxin-sensitive 40 kDa α-subunit has been isolated from brain (*41*) and shown to be structurally and immunologically similar to $Gi\alpha_{40}$ from neutrophils and HL-60 cells (*21,22*). Reconstitution studies performed by adding Gi or Go prepared from brain to plasma membranes from HL-60 cells previously treated with pertussis toxin showed that they were equally effective in restoring fMet-Leu-Phe plus GTP-stimulated formation of inositol phosphates (*42*). Addition of Go or Gi also restored bradykinin-dependent activation of GTPase and inhibition of adenylate cyclase in membranes from NG108-15 cells treated with pertussis toxin (*43*). Furthermore, Go or Gi stimulated the activity of partially purified phospholipase C from platelet membranes (*44*). These results suggest an apparent promiscuity of G-protein coupling to phospholipase C, but since the preparations of Go or Gi used in these experiments probably contained $G\alpha_{40}$, it seems more likely that this G-protein, which also stimulates phospholipase C activity (*44*), provides specificity for G-protein-phospholipase C coupling.

A different pertussis toxin target protein with an apparent molecular mass of 43 kDa has been partially purified from human erythrocytes (*45*). This protein has the properties expected for a G-protein α-subunit and is also present in membranes from brain, GH_4C_1 pituitary cells, leukocytes, and liver (*45*). A 54 kDa protein that was ADP-ribosylated by pertussis toxin and that copurified with an inositol lipid specific phospholipase C has been identified in the cytosolic fraction of thymocytes (*31*), while a pertussis toxin insensitive GTP-binding protein was found associated with purified vasopressin receptors in rat liver (*28,29*). Some indirect evidence suggests the possibility that an unidentified pertussis toxin sensitive G-protein may be involved in inhibiting phospholipase C (*32,46,47*). Several members of a group of G-proteins with Mr values betwen 20,000 and 25,000, including the well-characterized p21 *ras* oncogene product (*48*), have been purified but their possible function in relation to inositol lipid metabolism has not been elucidated (see *49*).

In summary, growing evidence suggests that several different G-proteins are responsible for activating phospholipase C. A number of novel G-protein α-subunits have been identified by protein purification, and multiple forms of Gi- and Go-like α-subunits with high-sequence homology have been identified by cDNA cloning (*20,21*) and by reaction with different antibodies (*22,50,51*). These studies also indicate that the $Gi\alpha_{41}$ that couples with receptors to inhibit adenylate cyclase activity is unlikely to activate phospholipase C. A new G-protein α-subunit has been identified from molecular cloning of cDNA, which is Gi-like but has a lower sequence homology than other known G-protein α-subunits. This subunit is particularly interesting because it lacks the cysteine site at the carboxyl terminus for ADP-ribosylation by pertussis toxin (*52,53*). If this G-protein is found to be widely expressed, it may account for pertussis toxin insensitivity of receptor coupling to phospholipase C observed in many tissues.

Phospholipases as G-Protein Effector Enzymes

Several inositol lipid-specific phospholipase C enzymes have been found in both the soluble and plasma membrane fractions of the cell (*53*). Membrane-bound phospholipase C has been purified after detergent solubilization from brain, with a molecular mass of 150 to 154 kDa (*55,56*); 61 kDa and 62 kDa enzymes have been purified from platelets (*57*) and uterus (*58*), respectively. Molecular cloning and determination of the complete amino acid sequence has been achieved for a number of phosphatidylinositol-specific phospholipase C enzymes from brain (*59-62*) and uterus (*63*), which show a remarkable lack of sequence homology. Generally, these enzymes will hydrolyse phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PIP), and PIP_2 at high Ca^{2+} concentrations, but they are more active toward PIP and PIP_2 at CA^{2+} concentrations below 10 μM. Forms of

phospholipase C, apparently specific for PI and not for polyphosphoinositides, have also been recognized (*64,65*). As shown in intact membrane preparations from a number of cell types, the major effect of activated G-protein on PIP_2-specific phospholipase C activity is to decrease the Ca^{2+} concentration required for enzyme activity to the physiological level of about 0.1 μM (*66–69*). In platelets, where most of the phospholipase C activity is recoverable from the soluble cell fraction, the partially purified enzyme is stimulated by GTPγS (*70–72*). In contrast, soluble phospholipase C from liver is not stimulated by GTPγS (*69*). At present, it remains an open question whether or not G-protein α-subunits in some tissues may dissociate from the membrane after receptor activation and, subsequently, interact with intracellular effector enzymes (*73*).

Some tissues also contain a phospholipase C specific for phosphatidylcholine, which appears to be coupled to receptors by an unidentified G-protein (*74,75*). Other types of phospholipase C specific for a PI-glycan structure involved in membrane-anchored proteins and insulin action have been described [see (*76*) for review]. Since diacylglycerol is produced by the action of all the different types of phospholipase C, it is evident that protein kinase C can be activated in the absence of Ins 1,4,5-P_3-induced Ca^{2+} mobilization.

G-proteins also control the hydrolysis of other lipids by coupling to phospholipase D (*77,78*) and phospholipase A_2 (*79–81*), which produce phosphatidic acid and arachidonic acid, respectively. Of the range of products from phospholipid hydrolysis, only Ins 1,4,5-P_3 is firmly established as a physiological messenger for Ca^{2+} release, although future studies may clarify the roles postulated for arachidonic acid (*82*) and phosphatidic acid (*77*) as Ca^{2+}-releasing agents. However, further metabolism of arachidonic acid to eicosenoids such as thromboxane A_2 (*33*) and the C6-sulfidopeptide leukotrienes (*83*) causes Ca^{2+} mobilization by their receptor coupling to phospholipase C. It thus appears that cells contain a heterogeneity of phospholipase effector enzymes as well as G-proteins, which further suggests the possibility that chemical signaling from different receptors is uniquely channeled to specific effector enzymes.

Regulation of Ins 1,4,5-P_3 Production

In the intact cell Ins 1,4,5-P_3 is formed together with small amounts of the 1,2 cyclic isomer only by phospholipase C-mediated hydrolysis of PIP_2 (see *84* for review). The amount of PIP_2 that is available for hydrolysis is only sufficient to maintain Ins 1,4,5-P_3 production for a few minutes. However, this hormone-sensitive PIP_2 pool is rapidly replenished via PIP from part of the much large PI pool by the action of PI and PIP kinases (*11,12*). These enzymes exist in multiple forms, and there is evidence that some types are activated by tyrosine kinases (*85*) or by protein kinase C (*86*). The large PI pool and the high activity of the enzymes synthesizing PIP_2 mitigates against the regulation of Ins 1,4,5-P_3 production by a limitation of substrate availability.

The production of Ins 1,4,5-P_3 is subject to several kinds of feedback regulation. A rapid inhibition of agonist-induced Ins 1,4,5-P_3 production and Ca^{2+} signaling is observed in many cell types by phorbol ester-induced activation of protein kinase C (*3,10,12,79*). This interaction can either be at the receptor or a post-receptor site. Some receptors, notably α_1-adrenergic and EGF receptors, are phosphorylated by protein kinase C with decreased agonist binding and prevention of agonist-mediated signal transduction to the G-proteins. (*87*). Phosphorylation of the α_1-adrenergic receptor by agonists has been demonstrated in DDT_1 MF-2 smooth muscle cells (*88*). The purified α_1-adrenergic receptor is also phosphorylated by protein kinase C (*89*). Receptor phosphorylation, however, does not seem to be a general mechanism for phorbol ester (PMA)-induced inhibition of agonist effects. Thus, an additional post-receptor site for protein kinase C-mediated interaction is suggested from studies with plasma membrane preparations. These showed no effect of PMA pretreatment on agonist binding affinity nor the ability of GTP analogues to suppress high-affinity agonist binding (*90*), but they did show an inhibitory effect on GTPγS-stimulated hydrolysis of PIP_2 (*90,91*). Since the production of Ins 1,4,5-P_3 as well as Ca^{2+} mobilization is inhibited by the activation of protein kinase C, the site for the post-receptor interaction is thought to be at the G-protein-phospholipase C complex. Although several G-protein α-subunits (notably $Gi\alpha_{41}$) have been shown to be phosphorylated by protein kinase C (*92,93*), clear evidence for the phosphorylation of an α-subunit functionally coupled to phospholipase C is presently lacking. Purified pholpholipase C from uterine smooth muscle can be phosphorylated by protein kinase C, but the ability to hydrolyze PIP_2 was unaffected (*58*). Resolution of the different mechanisms responsible for regulating Ins 1,4,5-P_3 production by activating protein kinase C will undoubtedly be aided by isolating and reconstituting functional receptor-G-protein phospholipase C complexes and using selective protein kinase C inhibitors in intact cells.

Regulation of Ins 1,4,5-P_3 Metabolism

Ins 1,4,5-P_3 is rapidly metabolized to products that do not release Ca^{2+} from intracellular stores. Recent studies concerned with elucidating the pathway of inositol phosphate metabolism have shown that Ins 1,4,5-P_3 is both dephosphorylated and further phosphorylated (*84*). Ins 1,4,5-P_3 is degraded to Ins 1,4-P_2 by both membrane-bound and soluble 5-phosphomonoesterases having K_m values of 3 to 18 μM (*94*), and it is also metabolized by a calmodulin-

dependent 3-kinase to Ins 1,3,4,5-P_4. The enzyme catalyzing this reaction has recently been purified from the soluble fraction of rat brain and has a K_m for Ins 1,4,5-P_3 of 0.2 to 0.4 μM (*95*). The concentration of Ins 1,4,5-P_3 in resting cells is equivalent to about 0.1 μM, although this may be mostly protein-bound, and it increases maximally during agonist stimulation to 1 to 2 μM (*96,97*). The low K_m of the Ins 1,4,5-P_3-kinase thus favors the formation of Ins 1,3,4,5-P_4.

The accumulation of Ins 1,4,5-P_3 in cells after agonist stimulation is typically biphasic, with a peak increase occurring within 10 to 15 sec, whereas the rate of PIP_2 hydrolysis is linear for several minutes. Two feedback effects increase the rate of Ins 1,4,5-P_3 metabolism. The increased cytosolic free Ca^{2+}caused by Ins 1,4,5-P_3-induced intracellular Ca^{2+} release promotes the formation of Ins 1,3,4,5-P_4 by Ca^{2+} activation of the calmodulin-dependent Ins 1,4,5-P_3 3-kinase (*95,98,99*). This enzyme appears to be activated by protein kinase C (*100*), while the 5-phosphomonoesterase has also been shown to be phosphorylated and activated by protein kinase C (*101*). However, there is some doubt whether or not the latter effect occurs in the intact cell, since the inhibition of protein kinase-C in platelets by staurosporine had no effect on thrombin-induced increases of inositol phosphates (*102*).

Ins 1,3,4,5-P_4 is hydrolyzed to Ins 1,3,4-P_3 by the membrane-bound 5-phosphomonesterase, and it competitively inhibits Ins 1,4,5-P_3 hydrolysis; Ins 1,3,4-P_3 can be both hydrolyzed (to Ins 3,4-P_2) by an inositol polyphosphate 1-phosphomonoesterase, and phosphorylated to Ins 1,3,4,6-P_4 by an ATP-dependent, calmodulin-insensitive 6-kinase (*84*). These inositol phosphates and their hydrolysis products have been shown to accumulate in various cells after agonist stimulation (*103,104*) with kinetics slower than those for Ins 1,4,5-P_3. The isomeric specificity of the inositol monophosphates formed are useful in characterizing relative flux through the different branches of the inositol phosphate metabolic pathway (*84,103*). Thus, Ins-1-P is formed primarily from the breakdown of PI; Ins-4-P is formed from the Ins 1,4-P_2 hydrolysis produced, either directly from PIP breakdown or from Ins 1,4,5-P_3 hydrolysis. Ins-3-P reflects degradation of Ins 1,3,4,5-P_4 and flux through the Ins 1,4,5-P_3 phosphorylation branch. Some of the inositol polyphosphate metabolites of Ins 1,4,5-P_3, notably Ins 1,3,4,5-P_4, may have second messenger functions, as described later.

Intracellular Ca^{2+} Mobilization

The Hormone-Sensitive Ca^{2+} Pool

Recent evaluations of the tissue distribution of total Ca^{2+} between the cytosol and the different organelles have led to the conclusion that the bulk of the sequestered Ca^{2+} is in the endoplasmic reticulum (*105,106*). It is also generally accepted that this pool represents the source of the Ca^{2+} released by Ca^{2+}-mobilizing agonists. However, the endoplasmic reticular structures are themselves heterogeneous, as are the pools of sequestered Ca^{2+}. This is revealed by the fact that, in numerous studies after adding agonists to intact cells or Ins 1,4,5-P_3 to permeabilized cells and isolated microsomal preparations, only a fraction of the sequestered Ca^{2+} is released (*10*). Recent studies have, in fact, suggested that a morphologically discrete organelle termed the calciosome, which generally copurifies with endoplasmic reticulum membranes, may represent the source of hormone releasable Ca^{2+} in nonmuscle cells (*107*). In some cells, such as smooth muscle (*108*), and Limulus photoreceptors (*109*), the sites for Ca^{2+} release may be adjacent to the plasma membrane. In Xenopus oocytes, both shallow and deep injections of Ins 1,4,5-P_3 caused a local release of Ca^{2+}, suggesting that Ins 1,4,5-P_3-sensitive Ca^{2+} pools are distributed throughout the cell (*110,111*). For most cell types, however, the distribution and morphology of Ins 1,4,5-P_3-sensitive Ca^{2+} pools have not been established. The mitochondria are not involved in Ins 1,4,5-P_3-mediated Ca^{2+} release, but respond to the increased cytosolic free Ca^{2+} by a net uptake of Ca^{2+} and an increase of the matrix-free Ca^{2+} concentration with consequent activation of mitochondrial dehydrogenases (e.g., α-ketoglutarate dehydrogenase) and respiration (*112,113*).

Sequestration of Ca^{2+} by the endoplasmic reticulum occurs by an ATP-driven Ca^{2+} pump. A high-affinity Ca^{2+}-ATPase with a molecular mass of 116 kDa and a K_m for Ca^{2+} of 0.1 to 0.2 μM has been purified from liver (*114*) and other tissues (*106*). This enzyme belongs to the class of $E_1P.E_2P$-ATPases and is biochemically, kinetically, and immunologically similar to the enzyme in skeletal muscle sarcoplasmic reticulum (*115*). In some cell types it is also calmodulin-dependent (*106*). As with sarcoplasmic reticulum, the Ca^{2+} sequestered by the endoplasmic reticulum is in equilibrium between free and bound forms. It is of considerable interest that calsequestrin (the major Ca^{2+}-binding protein of sarcoplasmic reticulum with a K_d for Ca^{2+} of 100 μM) or a similar protein has recently been detected in the endoplasmic reticulum of nonmuscle cells (*115*), including the calciosome (*107*). The total amount of Ca^{2+}-binding protein in the vesicular compartments presumably provides a limit to the amount of Ca^{2+} that can be accumulated. The free Ca^{2+} concentration in the endoplasmic reticulum has not been measured directly, but is probably two to three orders of magnitude higher than that in the cytosol, in order to provide a suitable electrochemical gradient for rapid Ca^{2+} efflux via opening of the Ins 1,4,5-P_3-sensitive Ca^{2+} channel. The rate of Ca^{2+} uptake into liver microsomes is inhibited by the intravesicular Ca^{2+} concentration with an apparent K_i of 250 to 300 μM (*116*), presumably by inhibiting the dissociation of Ca^{2+} from the phosphoenzyme. This effect probably accounts for the stimulation of MgATP-dependent Ca^{2+} accumulation into liver

microsomes by glucose 6-phosphate, which on hydrolysis with liberation of inorganic phosphate in the lumen, augments the intravesicular Ca^{2+}-buffering capacity (*117*).

The endoplasmic reticulum Ca^{2+} pump of pancreatic acinar cells has been shown to be activated after hormonal stimulation with a two fold increase of V_{max} for Ca^{2+} uptake and a decrease of the K_d for Ca^{2+} from 0.26 to 0.09 μM (*118*). Evidence obtained with saponized platelets suggests that the activation of protein kinase C may account for a similar stimulation of Ca^{2+} sequestration after thrombin addition (*119*). If the Ins 1,4,5-P_3-sensitive Ca^{2+} pool is distributed throughout the cell in discrete vesicular compartments, it is expected that with submaximum agonist stimulation, there would be a heterogeneous population of Ca^{2+}-depleted and nondepleted vesicles, depending on their spatial location and possible gradients of Ins 1,4,5-P_3 concentration within the cell. Therefore, an activation of the Ca^{2+} pump by either a Ca^{2+}/calmodulin mechanism or by a protein kinase C-mediated phosphorylation may augment the Ca^{2+} content of both Ins 1,4,5-P_3-sensitive and Ca^{2+}-insensitive pools. Since the activation of the Ca^{2+} pump by the protein kinase C mechanism could persist after the initial Ca^{2+} transient, the Ca^{2+} pools may be refilled from the extracellular Ca^{2+} pool without a simultaneous increase of the cytosolic free Ca^{2+}, as occurs when an agonist is displaced from its receptor (*120–124*).

Ins 1,4,5-P_3-Induced Ca^{2+} Release

The ability of Ins 1,4,5-P_3 to release Ca^{2+} from intracellular, nonmitochondrial, vesicular stores appears to be a ubiquitous property of cells. Its role in activating many hormonal responses and in stimulus-secretion coupling is firmly established (*10*). It is also involved in excitation-contraction coupling in smooth muscle, but whether Ins 1,4,5-P_3 is similarly involved in skeletal muscle is doubtful (*125*). The amount of Ca^{2+} released by maximal concentrations of Ins 1,4,5-P_3 is variable with different cell types, being 20 to 50% of the total cellular Ca^{2+} content (*10,126*). However, the increase of the cytosolic free Ca^{2+} is limited to about 1 μM because of the presence of a variety of Ca^{2+}-binding proteins in the cytosol (*106*). The Ca^{2+}-buffering capacity in the cytosol of neutrophils, for instance, has been estimated to be 0.76 mM, with an average dissociation constant of 0.55 μM (*127*). The peak increase of the cytosolic free Ca^{2+} occurs within a few seconds of its initiation.

Except for one report (*123*), the formation of Ins 1,4,5-P_3 has been shown to occur prior to or simultaneously with the increase of cytosolic free Ca^{2+} after addition of agonists to intact cells (*10,12*). It is important to note, however, that the density of certain receptor types in a given cell may be so high that agonist stimulation causes an accumulation of Ins 1,4,5-P_3 far in excess of the amount required for a maximal release of Ca^{2+}. Thus, with vasopressin stimulation of of hepatocytes, the peak increase of Ins 1,4,5-P_3 accumulation occurs after the peak of the cytosolic free Ca^{2+} (*12*), whereas in other cells [e.g., A431 carcinoma (*129*)], the accumulation of Ins 1,4,5-P_3 is more transitory and appears to correlate with a transient rise and fall of the cytosolic free Ca^{2+}. Studies using a stopped-flow measurement of the cytosolic free Ca^{2+} with the fluorescent Ca^{2+} indicator Fura-2 after agonist addition in parotid acinar cells (*130*) and platelets (*131*) have shown a lag of 100 to 200 msec before the onset of the Ca^{2+} increase. This lag is consistent with a required short delay in the sequence of steps involved in generating Ins 1,4,5-P_3. Measurement of Ca^{2+} changes with Fura-2 in groups of single hepatocytes by fluorescent videomicroscopy showed that individual cells responded to vasopressin or α_1-adrenergic stimulation only after a variable latent period of up to 45 sec, which was diminished by higher agonist concentrations (*132*). A similar effect has been observed in single adrenal glomerulosa cells after addition of angiotension II (*133*). These data are consistent with a requirement for the accumulation of a threshold concentration of a mediator in the signaling pathway, possibly determined by a variable density of receptors in each cell.

Investigations concerning the mechanism of Ins 1,4,5-P_3-induced Ca^{2+} release have provided evidence in favor of a ligand-gated Ca^{2+} channel, with the charge imbalance associated with Ca^{2+} efflux from the vesicular pool being compensated by an influx of K^+ through tetraethylammonium-sensitive K^+ channels (*134–136*). Further evidence for a Ca^{2+} channel rather than a cation-exchange mechanism is provided by the temperature insensitivity of Ins 1,4,5-P_3-activated Ca^{2+} efflux, as measured with permeabilized cells or microsomal fractions (*134,137,138*). More direct evidence for an Ins 1,4,5-P_3-gated Ca^{2+} channel has been obtained from electrophysiological studies after incorporation of microsomal proteins into lipid bilayers at the tips of patch-clamped micropipettes (*140*). Currently, the nature and properties of the reconstituted Ca^{2+} channels need to be defined, and it is not known if Ins 1,4,5-P_3 has any effect on microsomal K^+-channel activity.

Studies with permeabilized basophilic leukemia cells, in which the rate of Ins 1,4,5-P_3-mediated Ca^{2+} release was measured with Fura-2, have indicated that channel opening was sensitive to Ins 1,4,5-P_3 at concentrations below 10 nM and was highly cooperative (*141*). Furthermore, the Ins 1,4,5-P_3-mediated Ca^{2+} release mechanism is inhibited by sulfhydryl reagents, heparin, and the Ca^{2+} channel blockers—cinnarizine and flunarizine—but not by dantrolene, nifedipine, verapamil, diltiazem, or TMB-8 (*134,142*). The release of Ca^{2+} by Ins 1,4,5-P_3 is not affected by vanadate or quercetin, which inhibit the Ca^{2+} ATPase, or by caffeine, and it is independent of the presence of ATP (*143*). A photoaffinity derivative of Ins 1,4,5-P_3 has been shown to inhibit Ins 1,4,5-P_3-

induced Ca^{2+} release in permeabilized macrophages (*144*), while a monoclonal antibody raised against platelet microsomal proteins inhibited thrombin (plus GTP) and Ins 1,4,5-P_3-induced Ca^{2+} release in mixed dense tubular system vesicles and plasma membranes from platelets (*145*). As studied in permeabilized cells, Ins 1,4,5-P_3-mediated Ca^{2+} release does not become desensitized (*126,146*).

In preparations of microsomes from some tissues, notably liver (*141*), addition of Ins 1,4,5-P_3 produces a negligible release of Ca^{2+}, although a large fraction of the sequestered Ca^{2+} can be released by Ins 1,4,5-P_3 in microsomal fractions from other tissues (*146–148*) and saponin-permeabilized hepatocytes (*149*). Dawson (*150*) first showed that the Ins 1,4,5-P_3-stimulated Ca^{2+} release by liver microsomes was greatly enhanced by an addition of polyethylene glycol and low concentrations of GTP. It subsequently became evident that GTP itself promoted Ca^{2+} release with variable facilitation by polyethylene glycol and by a separate mechanism from Ins 1,4,5-P_3-induced Ca^{2+} release (*137,151–154*). GTP has been reported to promote fusion between microsomal vesicles, which correlated with the rate of GTP-mediated Ca^{2+} release (*151*). On the other hand, Nicchitta et al. (*153*) showed that GTP induced a nonspecific permeability of the liver microsomal membrane. The GTP effect on Ca^{2+} release in liver microsomes is associated with a polyethylene glycol-promoted increase of GTPase activity (*155*), suggesting the involvement of a GTP-binding protein. In contrast to the effects of GTPγS on G-proteins involved in receptor signaling, GTPγS inhibits the GTPase activity and GTP-mediated Ca^{2+} release (*155*).

Experiments with permeabilized neuronal and smooth muscle cell lines showed that Ins 1,4,5-P_3 released Ca^{2+} from the same pool as GTP, while GTP also released Ca^{2+} from an additional nonmitochondrial pool (*156*). In further studies, Gill and his colleagues suggested that uptake of Ca^{2+} into the Ins 1,4,5-P_3-sensitive Ca^{2+} pool is controlled by a GTP-regulated Ca^{2+}-translocating mechanism (*157*). In permeabilized hepatocytes, under certain conditions GTP enhanced the Ins 1,4,5-P_3-induced Ca^{2+} release (*158*). At present, the physiological significance of the GTP effect is not clear. Although it is unlikely that GTP activates a specific Ca^{2+} channel, it is possible that through the mediation of an intracellular GTP-binding protein, the GTP may unmask Ins 1,4,5-P_3-binding sites, increase the size of the Ins 1,4,5-P_3-sensitive Ca^{2+} pool, or be involved in the translocation of the calciosomes closer to the plasma membrane where Ins 1,4,5-P_3 is generated.

In a number of studies using membrane fractions derived from liver, adrenal cortex, macrophages, neutrophils, anterior pituitary, and brain (*159,160*) the density and affinity of Ins 1,4,5-P_3 binding sites have been characterized. Several reports indicate that plasma membrane-enriched fractions of liver (*159*) and platelets (*161*) contain a higher density of Ins 1,4,5-P_3-binding sites than do fractions derived from the endoplasmic reticulum. These findings are consistent with the proposal that Ins 1,4,5-P_3-binding and Ca^{2+} release occur in specialized organelles (calciosomes) that are not physically or functionally part of either the plasma or endoplasmic reticular membranes. The specificity of different inositol phosphates to displace radiolabeled Ins 1,4,5-P_3 from these binding sites is similar to their relative potency in causing Ca^{2+} release, namely Ins 1,4,5-P_3 > Ins 2,4,5-P_3 > Ins 4,5-P_2 > Ins 1,4-P_2, Ins P_5, Ins P_6. Other inositol phosphates such as Ins 1,3,4-P_3 and Ins 1,3,4,5-P_4 were 1 to 5% as effective as Ins 1,4,5-P_3 in causing a displacement of bound Ins 1,4,5-P_3. The number of binding sites for Ins 1,4,5-P_3 is higher in the cerebellum, compared with other parts of the brain (*162*), and is about 100 times greater than that observed in peripheral tissues. The half-maximum binding (40–80 nM) in cerebellar membranes (*160*) is also higher than values of 1 to 10 nM reported in other tissues (*159*). Thus, there is an apparent, large discrepancy between these low values for half maximum Ins 1,4,5-P_3 binding and the half maximum concentration of Ins 1,4,5-P_3 (0.1 to 2 μM) required to release Ca^{2+} from permeabilized cells or isolated microsomes (*10*). Several factors are known to affect the Ins 1,4,5-P_3 binding affinity that may account for this apparent discrepancy. Notably, Ins 1,4,5-P_3 binding is lower at physiological pH than at the more alkaline pH values typically used for binding studies (*160*) and is also inhibited by ATP (*159*). Furthermore, the transient nature of the Ca^{2+} release elicited by Ins 1,4,5-P_3 in permeabilized cells and membrane preparations reflects its further metabolism to relatively inactive products, so that the Ins 1,4,5-P_3 concentrations present in the medium might be overestimated.

The Ins 1,4,5-P_3 receptor has recently been purified from rat cerebellum using a heparin affinity column (*163*). This solubilized membrane protein was shown to have a molecular mass of 260 kDa by SDS-PAGE and to bind Ins 1,4,5-P_3 with a K_d of 100 nM at pH 8.3. In contrast to studies with cerebellar membrane fractions, where Ca^{2+} was shown to inhibit Ins 1,4,5-P_3 binding with a half-maximal effect at 300 nM (*160*), the binding of Ins 1,4,5-P_3 to the purified receptor was unaffected by Ca^{2+} (*163*). The Ca^{2+} sensitivity is apparently conferred by a separate protein present in solubilized brain membranes having an estimated Mr of 300,000 by gel filtration (*164*). The effect of Ca^{2+} in cerebellar membranes is to decrease the affinity of Ins 1,4,5-P_3 binding to the receptor (*165*). An increase of extravesicular Ca^{2+} in the range from 1 to 10 μM has been shown to inhibit Ins 1,4,5-P_3-induced Ca^{2+} release in permeabilized neuronal cells and microsomes (*137,166*). In studies with cerebellar microsomes, the concentration of Ca^{2+} required for inhibition was lower at suboptimal Ins 1,4,5-P_3 concentrations (*165*). Feedback inhibition by Ca^{2+} to decrease the affinity of Ins 1,4,5-P_3 binding to the receptor might provide a mechanism for generating

oscillations in cytosolic Ca^{2+} concentration without changes in Ins 1,4,5-P_3 concentration. (See later section.)

Ca^{2+} Efflux from the Cell

An early event following stimulation of cells by Ca^{2+}-mobilizing agonists is an increased efflux of Ca^{2+}, with a consequent fall in the total cellular Ca^{2+} content (*1,2,16*). Evidence with hepatocytes has indicated that the onset of net Ca^{2+} efflux is delayed by 5 to 10 sec after agonist addition, indicating that there is an initial inhibition of the plasma membrane Ca^{2+} efflux pump (*167*). The mechanism of this effect has not been ascertained, but it may relate to the altered inositol lipid environment of the Ca^{2+}-ATPase (*168*) or to a direct inhibitory effect of Ins 1,4,5-P_3 (*169*). Subsequently, however, this inhibition is overcome so that Ca^{2+} efflux is stimulated above the resting rate. This can be attributed partly to the Ins 1,4,5-P_3-induced increase of cytosolic free Ca^{2+}, which stimulates the calmodulin-dependent plasma membrane Ca^{2+}-ATPase and the electrogenic Na^+/Ca^{2+} exchanger. The latter system is present in the plasma membrane of many cells (*170*). The removal of extracellular Na^+ has been shown to inhibit agonist-stimulated Ca^{2+} efflux in some cells such as arterial smooth muscle (*171*), but not in others, such as platelets (*172*). However, in most cells, the efflux of Ca^{2+} by the Ca^{2+}-ATPase probably predominates. A net loss of Ca^{2+} from the cell occurs over the first few minutes until there is a balance between the rate of Ca^{2+} efflux and the agonist-stimulated rate of Ca^{2+} influx (*122*). The loss of Ca^{2+} from the Ins 1,4,5-P_3-sensitive Ca^{2+} pool makes the cell unresponsive to further Ca^{2+} mobilization by additional agonist stimulation until the pool is refilled from the extracellular medium.

The Ca^{2+} efflux rate is not solely dependent on the cytosolic free Ca^{2+} concentration since the rate of exchange of Ca^{2+} across the plasma membrane is increased to 2- to 4-fold during the sustained phase of the Ca^{2+} transient when the cytosolic free Ca^{2+} is only slightly elevated above control levels (*1*). Studies of a variety of cells have indicated that activation of protein kinase C by phorbol esters stimulates Ca^{2+} efflux (*172–174*). The mechanism of this effect has been investigated by studies with the erythrocyte Ca^{2+}-ATPase, which showed that calmodulin increased the V_{max} 14-fold with a 4-fold decrease of the K_m to 0.3 μM, while the addition of activated protein kinase C independently increased the V_{max} by 5- to 7-fold without changing the K_m (*175*).

A Ca^{2+}-independent activation of the Ca^{2+}-ATPase by protein kinase C may thus account for the higher Ca^{2+} efflux rate in cells after prolonged agonist stimulation compared with control cells, despite the small elevation of cytosolic free Ca^{2+}. This dual regulation of the Ca^{2+}-ATPase by Ca^{2+}/calmodulin and by protein kinase C, together with different contributions of the Na^+/Ca^{2+} exchanger to Ca^{2+} efflux, probably accounts for the many detailed differences in the relative duration and shape of the Ca^{2+} transient observed upon addition of different agonists to various cell types.

Extracellular Ca^{2+} Mobilization

A sustained hormonal response requires an influx of extracellular Ca^{2+} to maintain an elevated cytosolic free Ca^{2+} concentration. Evidence for an agonist dependent stimulation of Ca^{2+} influx is based on the dependence of the sustained phase upon extracellular Ca^{2+} and an increase of unidirectional $^{45}Ca^{2+}$ influx (*1,2*). The temporal relationship between intracellular Ca^{2+} release and stimulated influx appears cell specific, since rapid kinetic measurements show that the onset of stimulated Ca^{2+} influx occurs at least as rapidly as intracellular Ca^{2+} release in parotid cells (*130*) and platelets (*131*). In A10 smooth muscle (*132*) and GH_3 pituitary (*176*) cells, stimulated Ca^{2+} influx occurs after the transient intracellular Ca^{2+} release.

There is a 10,000-fold concentration gradient of Ca^{2+} across the plasma membrane, which requires a strict regulation of its permeability to Ca^{2+}. Several types of voltage-sensitive Ca^{2+} channels that open with membrane depolarization have been well characterized (*177*). In some secretory cells—notably GH_3 pituitary (*176*) and adrenal glomerulosa cells (*121*)—Ca^{2+} entry by voltage-sensitive Ca^{2+} channels may contribute towards the sustained phase of the agonist-induced Ca^{2+} transient. However, in other cells such as hepatocytes (*178*), the response to Ca^{2+} mobilizing agonists may be associated with a hyperpolarization because of a secondary Ca^{2+} activation of K^+ and Cl^- channels. Alternatively, agonist-induced Ca^{2+} entry is considered to be regulated by a process usually referred to as a receptor-operated Ca^{2+} channel. This entry of Ca^{2+} is only inhibited by agents that block voltage-sensitive Ca^{2+} channels when they are added at very high concentrations (*120*). In fact, several mechanisms may mediate activation of Ca^{2+} channels by receptor occupation. One mechanism is by direct ligand activation, which appears to occur with the ATP-activated high conductance cation channel in smooth muscle (*179*). Another mechanism is by receptor-activated G-proteins, which have been demonstrated to regulate several types of K^+ and Ca^{2+} channels in a variety of cells (reviewed in *180*). Evidence consistent with a role for a G-protein in activating receptor operated Ca^{2+} channels is suggested from the observations that pretreatment with pertussis toxin inhibited Ca^{2+} influx, which was induced by vasopressin in hepatocytes (*181*) and by angiotensin II in adrenal glomerulosa cells (*182*), but did not affect intracellular Ca^{2+} mobilization. A nonselective cation channel activated by a rise of cytosolic free Ca^{2+} has been reported (*183,184*), but in most cells the onset of Ca^{2+} influx does not lag behind intracellular Ca^{2+} mobilization. Stimulated Ca^{2+}

influx persists after the initial Ca^{2+} transient, and it is dependent on receptor occupancy, rather than on the cytosolic free Ca^{2+} concentration (*120,121,123,185*).

An attractive possibility that has generated considerable attention is that Ins 1,4,5-P_3, itself, or one of its metabolic products may be responsible for agonist-stimulated Ca^{2+} influx. Ins 1,4,5-P_3-induced Ca^{2+} release has been demonstrated in Na^+-loaded plasma membrane vesicles from platelets (*161*). Mitogenic stimulation of T lymphocytes has been shown to be associated with activation of a low conductance voltage-independent Ca^{2+} channel in the cell-attached patch, which could be directly activated by Ins 1,4,5-P_3 after excision of the patch (*186*).

An involvement of Ins 1,3,4,5-P_4 in mediating Ca^{2+} influx was first suggested from microinjection experiments with sea urchin eggs (*187*). Further studies showed that there was no effect of Ins 1,3,4,5-P_4 on Ca^{2+} influx, but at high concentrations, it induced release of intracellular Ca^{2+} (*188*). Experiments with Xenopus oocytes showed that an injection of Ins 1,3,4,5-P_4stimulated an oscillatory Ca^{2+}-activated chloride current at concentrations 20-fold greater than those required for Ins 1,4,5-P_3 to elicit a similar response and also activated a voltage-sensitive Ca^{2+} channel, particularly after a priming injection of Ins 1,4,5-P_3 (*189*). Measurements of whole-cell current with voltage-clamped lacrimal acinar cells (*190*), where acetylcholine in the presence of extracellular Ca^{2+} evokes a sustained hyperpolarization of the plasma membrane by increasing an outward Ca^{2+}-activated K^+ current, showed that the sustained response required the presence of both Ins 1,4,5-P_3 and Ins 1,3,4,5-P_4 as well as extracellular Ca^{2+}. In other studies, an inward current due to nonspecific cation channels was evoked by injection of Ins 1,3,4,5-P_4 or Ins 1,3,4-P_3 into NG108-15 neuronal cells. Unlike Ins 1,4,5-P_3 injection, these compounds failed to elicit the outward Ca^{2+}-activated K^+ (*191*). In contrast, studies with NIE-115 neuroblastoma cells showed that injection of Ins 1,3,4,-P_3, but not Ins 1,3,4,5-P_4, produced a membrane depolarization in 50% of the cells tested (*192*).

In summary, it must be concluded that there is insufficient evidence to deduce the mechanism for receptor-dependent Ca^{2+} entry into cells, although it is likely that different mechanisms may predominate in different cell types. Whether Ins 1,3,4,5-P_4 or another inositol phosphate are involved remains an open question. Specific Ins 1,3,4,5-P_4 binding sites have been demonstrated in membranes of several tissues that are distinct from Ins 1,4,5-P_3 binding sites (*193,194*), but their function has not yet been ascertained.

Oscillations in Cytosolic Free Ca^{2+}

In contrast to the many studies with bulk cell suspensions where agonist-induced Ca^{2+} transients are typically biphasic, the development of methods for measuring changes of Ca^{2+} in single cells has revealed that the response may be oscillatory. Measurements with the bioluminescent protein, aequorin, in single hepatocytes stimulated with agonist showed a series of periodic increases in cytosolic free Ca^{2+} to above 600 nM, each returning to resting levels within 7 sec (*195,196*). The frequency of these oscillations was a function of phenylephrine concentration, with a period varying from 20 to 240 sec. Low concentrations of vasopressin and angiotensin II, which produce nonsaturating amounts of Ins 1,4,5-P_3, caused similar oscillations, suggesting that the occurrence of Ca^{2+} oscillations is favored when tissue Ins 1,4,5-P_3 concentrations are low. In contrast, Fura-2 loaded hepatocytes rarely exhibit Ca^{2+} oscillations after addition of agonists (*132*). However, in other cell types, oscillations of the cytosolic free Ca^{2+} in a proportion of cells have been observed using Fura-2 as the Ca^{2+} indicator. For example, BC3H-1 cells stimulated with phenylephrine exhibited a burst of Ca^{2+} spikes that ceased after several minutes (*197*), low concentrations of angiotensin II-elicited Ca^{2+} oscillations in adrenal glomerulosa cells (*133*) and macrophages induced to spread onto a suitable substratum (frustrated phagocytosis) showed Ca^{2+} oscillations (*198*). In parotid acinar cells a damped series of oscillations could be evoked by carbachol, but in a concentration-independent manner (*199*); rat peritoneal mast cells stimulated with antigen responded variably with one, two, or more spikes of increased Ca^{2+} (*200*). Similarly, the addition of carbamylcholine to insulinoma HIT (T-15) cells loaded with Fura-2 produced a series of characteristic but damped Ca^{2+} oscillations in each responding cell (*201*). In general, measurements of Ca^{2+} with Fura-2 show a more transient, irregular pattern of Ca^{2+} fluctuations than those obtained with aequorin and with a smaller proportion of cells generating oscillations.

The difference between the data obtained with aequorin and Fura-2 even in the same cell type, suggest that they may arise from the different properties of the Ca^{2+} indicators. Since Fura-2 is a Ca^{2+} chelator, it is possible that it diminishes the peak rise of the Ca^{2+} transients, which may be part of a negative feedback interaction responsible for generating the oscillations. On the other hand, the nonlinear relationship betwen aequorin bioluminescence and Ca^{2+} concentration may amplify spatially localized oscillations of Ca^{2+}. Thus, a series of transient, localized increases in Ca^{2+} might be detected by aequorin as large, transient spikes, decaying rapidly as Ca^{2+} diffuses into the remainder of the cytosol, whereas Fura-2 might not detect such a response because of its lower sensitivity to local changes of the Ca^{2+} concentration. However, sustained Ca^{2+} oscillations have been observed in oocytes after fertilization using either Ca^{2+}-selective microelectrodes (*202*) or aequorin to measure the Ca^{2+} changes (*203*).

Indirect evidence for oscillations of the cytosolic free Ca^{2+} in response to Ca^{2+}-mobilizing hormones has been obtained from electrophysiological measurements of the oscillatory behavior of Ca^{2+}-activated K^+ and Cl^- channels. In HeLa cells, histamine stimulated a pattern of Ca^{2+}-dependent channel activation, characterized by a series of bursts, which were separated by silent periods. These correlated with membrane potential oscillations and occurred in the absence of extracellular Ca^{2+} (*204*). Similarly, α_1-adrenergic stimulation of voltage-clamped guinea pig hepatocytes induced oscillatory membrane conductance increases to K^+ and Cl^-, which were attributed to Ca^{2+}-activated K^+ and Cl^- channels (*205*). A close correlation between oscillatory Ca^{2+}-activated K^+ channel activity and intracellular Ca^{2+} has been reported in hamster oocytes (*202*), with similar responses being observed after injection of GTPγS (*206*).

Mechanisms for the generation of Ca^{2+} oscillations appear to fall into two categories: oscillations that occur secondary to spontaneous action potentials, as in some secretory cells (*207*), and oscillations induced by Ca^{2+}-mobilizing hormones. The latter can be initiated in the absence of extracellular Ca^{2+}, indicating that intracellular Ca^{2+} release is the primary source of Ca^{2+}, although extracellular Ca^{2+} is required for their maintenance (*197,198,204,205*). A minimum oscillating system requires a feedback loop; for sustained oscillations, some delay step is required to generate the periodicity (*208*). Theoretically, oscillations in the Ins 1,4,5-P_3 concentration could be produced by a negative feedback effect on the receptor or the G-protein coupled to activation of phospholipase C by protein kinase C. The delay step could be caused by a slow rate of activation of the G-protein or of protein kinase C by diacylglycerol. However, such a mechanism involving a periodically activated protein kinase C is difficult to reconcile with the decrease in the frequency of the Ca^{2+} oscillations observed by addition of exogenous diacylglycerol or phorbol ester to hepatocytes (*209*). Furthermore, in guinea pig hepatocytes, oscillations in Ca^{2+}-dependent ion conductances could be induced by an injection of Ins 1,4,5-P_3, suggesting that changes in the Ins 1,4,5-P_3 concentration are not required (*205*). A possible mechanism for negative feedback is suggested by the inhibitory effect of Ca^{2+} on Ins 1,4,5-P_3 binding to its receptor, as described earlier. Also, the time required for reaccumulation of Ca^{2+} into the Ins 1,4,5-P_3-sensitive pool could provide the delay to generate a characteristic periodicity.

In summary, there is good evidence that some oscillatory Ca^{2+} response occurs in stimulated cells. Oscillations are only seen in single cells, since individual cells in suspensions respond asynchronously. Possible advantages of a periodic frequency-modulated signal, compared to a graded amplitude-modulated signal, include protection from desensitization or preventing mitochondrial Ca^{2+} overload (*196*). An alternative possiblility is suggested by an analogy with neural transmission where a self-propagating action potential allows transmission of information over distances. There is considerable evidence that cells are in communication with each other through gap junctions (*210*), which is also suggested by the synchronization of oscillations in the intact perfused liver (*211*). If oscillations at low frequency induced in one cell facilitate responses in adjacent cells, the overall tissue response would be increased. Should such a mechanism exist, the regulation of gap junction permeability by a variety of agents (*210*) would enable fine modulation of the sensitivity of the tissue response.

Summary

The involvement of inositol lipid metabolism in agonist-mediated Ca^{2+} signaling by Ins 1,4,5-P_3 has become firmly established. Recent advances have led to a better understanding of the proteins associated with signal transduction in the plasma membrane. A number of specific receptors, G-proteins, phospholipases, and inositol lipid kinases have now been purified and characterized. An Ins 1,4,5-P_3 receptor has also been purified, which is presumably involved in mediating the Ca^{2+} efflux from intracellular stores. The morphological site of the hormone-sensitive Ca^{2+} pool has been tentatively identified as discrete, specialized intracellular structures (calcisosomes); however, further studies are required to demonstrate that these contain Ins 1,4,5-P_3-gated Ca^{2+} channels. Receptor occupancy by Ca^{2+}-mobilizing agonists also stimulates Ca^{2+} entry into the cell, but the mechanism for the activation of voltage-insensitive Ca^{2+} channels and the possible involvement of Ins 1,4,5-P_3, Ins 1,3,4,5-P_4 and/or G-proteins in this process has not been established.

The Ca^{2+} signaling pathway is subject to multisite feedback regulation by Ca^{2+}, itself, and by a diacylglycerol-mediated activation of protein kinase C. Potential sites for Ca^{2+} interaction are displacement of Ins 1,4,5-P_3 from its receptor by a Ca^{2+}-dependent mechanism, promotion of Ins 1,3,4,5-P_4 formation by the Ca^{2+}/calmodulin-regulated Ins 1,4,5-P_3 3-kinase and efflux of Ca^{2+} from the cell, or sequestration into intracellular Ca^{2+} stores by Ca^{2+}/calmodulin-regulated Ca^{2+}-ATPases. Protein kinase C activation potentially affects the rate of generation of Ins 1,4,5-P_3 by negative feedback to the receptor-G-protein-phospholipase C transduction system and possibly also the rate of Ins 1,4,5-P_3 removal by activation of an inositol polyphosphate 5-phosphomonoesterase and/or Ins 1,4,5-P_3 3-kinase. It may also attentuate the Ca^{2+} transient directly by increasing the activity of Ca^{2+}-ATPases associated with the plasma membrane and the endoplasmic reticulum. Cell-to-cell heterogeneity in the relative control strengths of these different mechanisms may explain the differences in the Ca^{2+} signal in different tissues and even in different cells within a population. The ability of Ca^{2+} and

protein kinase C to provide negative feedback at various points in the signal transduction pathway suggests that a complex mechanism involving multiple feedback loops is likely to regulate the generation of Ca^{2+} oscillations seen in some cells. The precise interactions between these feedback mechanisms and the crosstalk with other intracellular signaling pathways enables each cell to respond in an appropriate and unique manner.

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